

Analysis of Human Immunodeficiency Virus Type 1 Reverse Transcriptase Subunit Structure/Function in the Context of Infectious Virions and Human Target Cells

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The reverse transcriptase (RT) of all retroviruses is required for synthesis of the viral DNA genome. The human immunodeficiency virus type 1 (HIV-1) RT exists as a heterodimer made up of 51-kDa and 66-kDa subunits. The crystal structure and in vitro biochemical analyses indicate that the p66 subunit of RT is primarily responsible for the enzyme's polymerase and RNase H activities. Since both the p51 and p66 subunits are generated from the same coding region, as part of the Pr160^{Gag-Pol} precursor protein, there are inherent limitations for studying subunit-specific function with intact provirus in a virologically relevant context. Our lab has recently described a novel system for studying the RT heterodimer (p51/p66) wherein a LTR-vpr-p51-IRES-p66 expression cassette provided in *trans* to an RT-deleted HIV-1 genome allows precise molecular analysis of the RT heterodimer. In this report, we describe in detail the specific approaches, alternative strategies, and pitfalls that may affect the application of this novel assay for analyzing RT subunit structure/function in infectious virions and human target cells. The ability to study HIV-1 RT subunit structure/function in a physiologically relevant context will advance our understanding of both RT and the process of reverse transcription. The study of antiretroviral drugs in a subunit-specific virologic context should provide new insights into drug resistance and viral fitness. Finally, we anticipate that this approach will help elucidate determinants that mediate p51-p66 subunit interactions, which is essential for structure-based drug design targeting RT heterodimerization.

Since the first cases of AIDS were reported in the early 1980s, this disease has killed over 20 million people worldwide. The human immunodeficiency virus (HIV), the causative agent of AIDS, is a complex retrovirus that reverse transcribes its RNA genome into double-stranded DNA upon infection of permissive host cells (see reference 8 for a review). The reverse transcription process is essential for virus infection and is catalyzed by the reverse transcriptase (RT) enzyme. Therefore, RT has been a critical target for the chemotherapeutic treatment of individuals infected with HIV (19).

Similar to other lentiviruses, the HIV type 1 (HIV-1) RT is encoded as part of the Gag-Pol precursor protein Pr160^{Gag-Pol}. During and after assembly of the virus particle, Pr160^{Gag-Pol} is cleaved by the viral protease (PR) to liberate a 66-kDa RT subunit. Subsequent cleavage of the C-terminal domain of p66 produces the 51-kDa RT subunit. The two different subunits dimerize in the virion and form the functional RT p51/p66 heterodimer (6). The structure of the HIV-1 RT heterodimer has been elucidated by X-ray crystallography in different configurations, including unliganded (39) and complexed with nucleoside RT inhibitors (NRTIs) (40), with nonnucleoside RT inhibitors (NNRTIs) (37, 46), with double-stranded DNA (15, 22), or with RNA-DNA templates (41). These studies show that p66 can be divided structurally into the polymerase

and RNase H domains. The polymerase domain is further divided into the fingers, palm, thumb, and connection subdomains (22). The relative arrangement of the subdomains is quite different in each of the subunits, and thus, the structures and functions of p51 and p66 are distinct. For example, the polymerase activity of this enzyme has been mapped solely to the larger p66 subunit (13, 23, 29). In the p51 subunit, the three aspartates (D110, D185, and D186) comprising the polymerase active site in p66 are buried (41), and the p51 subunit of the p51/p66 heterodimer does not catalyze DNA synthesis (29). The main hurdle for studying the individual RT subunits in the context of infectious virus is that both p51 and p66 are derived from the same coding region, and thus, any mutation in the polymerase domain occurs in both subunits of the RT heterodimer.

Anti-RT drugs can be grouped into NRTIs and NNRTIs. NRTIs mechanistically act as DNA chain terminators, while NNRTIs bind to a hydrophobic pocket close to, but distinct from, the RT active site in the p66 subunit. The emergence of drug-resistant HIV variants and serious side effects related to drug toxicities limit the efficacy of existing therapies (25). This emphasizes the need for new drugs active against drug-resistant mutants selected by current therapies and/or directed to novel targets in the viral replicative cycle (27). By exploiting the ability of HIV-1 Vpr to incorporate into virions via interaction with the p6 domain of the Gag precursor polyprotein (Pr55^{Gag}) (32), we developed a Vpr fusion protein-based strategy wherein a LTR-vpr-p51-IRES-p66 expression cassette provided in *trans* to an RT-deleted HIV-1 genome allows inde-

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TABLE 1. Plasmids used in this study

Plasmid	Abbreviation	Description
pSG3 ^{wt}	SG3	HIV-1 wild-type proviral clone
pSG3 ^{FN}	FN	SG3 with in-frame deletion in RT (see Materials and Methods)
pSG3 ^{M7}	M7	Does not express RT or IN; described in reference 29
pLR2P-vpr-p51-IRES-p66	<i>vpr-p51/p66</i>	Original <i>trans</i> -heterodimer expression plasmid (reference 29)
pLR2P-vpr- ^{30Pro} p51-IRES-p66	<i>vpr-^{30Pro}p51/p66</i>	<i>vpr-p51/p66</i> modified as described in Materials and Methods
pLR2P-vpr- ^{45Pro} p51-IRES-p66	<i>vpr-^{45Pro}p51/p66</i>	<i>vpr-p51/p66</i> modified as described in Materials and Methods
pLR2P-vpr- ^{60Pro} p51-IRES-p66	<i>vpr-^{60Pro}p51/p66</i>	<i>vpr-p51/p66</i> modified as described in Materials and Methods
pLR2P-vpr-p51-IRES-p66IN	<i>vpr-p51/p66IN</i>	IN expressed as p66IN fusion; hence, Vpr-IN can be excluded
pLR2P-vpr-Δp51-IRES-p66	<i>vpr-Δp51/p66</i>	Control for non-Vpr-p51-mediated virion incorporation of p66
pLR2P-vpr-p66	<i>vpr-p66</i>	Vpr-p66 expression plasmid
pLR2P-vpr-p51	<i>vpr-p51</i>	Vpr-p51 expression plasmid
pLR2P-p66	<i>p66</i>	p66 expression plasmid
pLR2P-vpr-IN	<i>vpr-IN</i>	Vpr-IN expression plasmid

pendent expression and analysis of the two RT subunits in a context that is physiologically relevant to HIV-1 replication (29). In this report, we describe in detail the specific approaches, pitfalls, and alternative strategies that shaped the development and affect the application of this functional assay system for analyzing RT subunit-specific structure/function. Additionally, we provide the underpinning for its utility in studying drugs that target the heterodimeric RT enzyme.

MATERIALS AND METHODS

Cells, antibodies, and antiviral drugs. The 293T and TZM-bl cell lines (49) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplement with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (0.1

mg/ml). Antibodies used included monoclonal antibodies (MAb) to HIV-1 CA (183-H12-5C; contributed by Bruce Chesebro and Hardy Chen) and HIV-1 RT and RNase H (8C4 and 7E5, respectively; contributed by Dag E. Helland), obtained through the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH. The NRTI (lamivudine [3TC]) and NNRTI (nevirapine [NVP]) were obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH. They were resuspended in dimethyl sulfoxide at a concentration of 1.0 mM and serially diluted to the desired concentrations.

HIV-1 proviral clones. The HIV-1 pSG3^{wt} proviral clone (SG3) (10) (GenBank accession no. L02317) was used to produce wild-type virus and to construct all proviral and recombinant RT and IN expression plasmids (plasmids used are listed in Table 1). The pSG3^{FN} (FN) clone was constructed using the strategy described by Dubay et al. (7) for the HXB2 pFN clone (Fig. 1A). Briefly, the FN

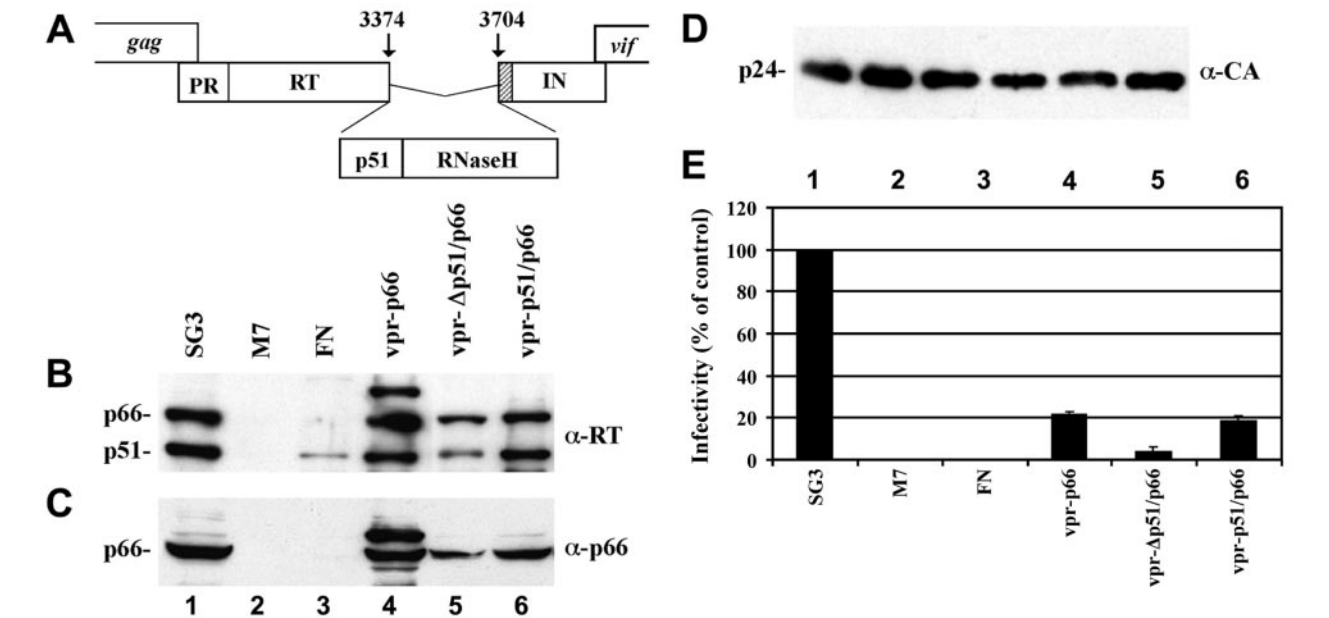


FIG. 1. Analysis of FN-derived virions containing *trans*-RT heterodimer. (A) Illustration of the FN proviral construct. This proviral construct was made from the wild-type SG3 plasmid using a previously described strategy (7). The clone contains a 110-amino-acid deletion (nucleotides 3374 to 3704) in the RT reading frame. Most of the RNase H domain and 13 amino acids of the carboxyl end of the polymerase domain were removed, leaving the IN coding region in frame. (B to D) Virion incorporation and proteolytic processing of *trans*-heterodimeric RT. The FN proviral DNA was transfected alone or cotransfected with the *vpr-p66*, *vpr-p51/p66*, or *vprΔp51/p66* expression plasmid, respectively. The wild-type SG3 and RT-IN-negative M7 proviruses were included as controls. The transfection-derived virions were concentrated by ultracentrifugation, lysed, and analyzed by immunoblotting using (B) anti-RT (α-RT), (C) anti-p66 (α-p66), or (D) anti-capsid (α-CA) MAb. (E) Analysis of infectivity. Transfection-derived viruses were analyzed for infectivity using the TZM-bl reporter cell line as described in Materials and Methods. Results are expressed as a percentage relative to an equal amount of wild-type SG3 virus.

clone contains an in-frame 110-amino-acid deletion and was created by Acc65I digestion to remove a 330-nucleotide fragment of the *pol* gene. The 5' overhang was filled using dGTP and the Klenow fragment of DNA polymerase. The remaining single-stranded regions were removed with S1 nuclease, and the plasmid was ligated. The deleted DNA segment encoded a large part of RNase H and 13 amino acids of the carboxyl end of the polymerase domain of RT. This clone encodes a truncated form of RT while maintaining the IN coding region in frame. The pSG3^{M7} (M7) proviral construct was created from pSG3^{S-RT} (S-RT) as described previously (29, 51).

Construction of heterodimeric RT expression plasmids. The pLR2P-vpr-p51-IRES-p66 (*vpr-p51/p66*) plasmid was constructed for independent expression of the RT subunits in *trans* (29). Since the molecular mass of the unprocessed Vpr-p51 fusion protein is very similar to that of p66, these two proteins are not distinguishable using antibody directed to the polymerase domain of RT. To allow differentiation between Vpr-p51 and p66 by molecular mass, three derivatives of the original *vpr-p51/p66* construct were made. These constructs were generated by including additional PR sequence 5' of the p51 coding region in *vpr-p51*. Either 90, 120, or 150 bp of PR sequence (encoding 30, 45, or 60 amino acids, respectively) was introduced at this position, generating *vpr-30Pro-p51/p66*, *vpr-45Pro-p51/p66*, or *vpr-60Pro-p51/p66*, respectively. The *vpr-p51/p66-IN* plasmid was constructed by cutting the pLR2P-vpr-RT-IN plasmid (51) with XmaI-XhoI and ligating the RT-IN fragment with XmaI-XhoI-cut *vpr-p51/p66*. The *vpr-Δp51/p66* control plasmid was constructed to contain a translational stop codon at the first amino acid position of p51. Other derivatives of *vpr-p51/p66* were constructed using PCR-based site-directed mutagenesis and cloning into the BglII-MluI or XmaI-XhoI sites of either p51 or p66, respectively. All clones were confirmed by nucleotide sequencing. The pLR2P-vprIN (*vpr-IN*) plasmid was described previously (51).

Transfections and analysis of virus infectivity. DNA transfections were performed on monolayer cultures of 293T cells grown in six-well plates using the calcium phosphate DNA precipitation method. Unless otherwise noted, each cell monolayer (well) was transfected with 6 μg of proviral DNA, 3 μg of the *vpr-p51/p66* plasmid constructs, and 1 μg of the *vpr-IN* plasmid. Culture supernatants from the 293T cells were collected 60 h posttransfection, clarified by low-speed centrifugation (1,000 × g, 10 min), and filtered through 0.45-μm-pore-size sterile filters. The clarified supernatants were analyzed for HIV-1 p24 antigen concentration by enzyme-linked immunosorbent assay (Beckman-Coulter Inc.).

Virus infectivity was assessed using the TZM-bl reporter cell line as described earlier (49). Briefly, virus-containing supernatants were normalized for p24 antigen concentration, serially diluted (fivefold dilutions), and used to infect monolayer cultures of TZM-bl cells. At 48 h postinfection, the cells were fixed and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) reagent as described earlier (21). The blue-stained cells were counted using a light microscope. Wells containing between 30 and 300 blue cells were used to calculate the number of infectious units of virus per nanogram of p24 antigen.

Western blot (immunoblot) analysis. Transfection-derived virions were concentrated by ultracentrifugation through a 20% sucrose cushion (125,000 × g, 2 h, 4°C) using an SW41 rotor (Beckman Inc.). Pellets were solubilized in loading buffer (62.5 mM Tris-HCl [pH 6.8], 0.2% sodium dodecyl sulfate, 5% 2-mercaptoethanol, 10% glycerol) and boiled, and proteins were separated on 12.0% polyacrylamide gels containing sodium dodecyl sulfate. Following electrophoresis, proteins were transferred to nitrocellulose (0.2-μm pore size) by electroblotting and incubated for 1 h at room temperature in blocking buffer (5% nonfat dry milk in phosphate-buffered saline). The blocked blot was exposed to the appropriate primary antibody for 1 h in blocking buffer with constant mixing. After extensive washing, bound antibodies were detected by chemiluminescence using horseradish peroxidase-conjugated, species-specific secondary antibodies (Southern Biotechnology Associates, Inc.) as described by the manufacturer (Amersham Biosciences).

Inhibition of *trans*-RT using NRTI and NNRTI. Virions were derived by cotransfection of 293T cells either with SG3 or with the combination of M7, *vpr-p51/p66*, and *vpr-IN*. TZM-bl cells were seeded overnight in 96-well plates at a concentration of 9,000 cells in 100 μl of medium per well. The culture medium was removed and replaced with 50 μl of DMEM containing 10% FBS and 2× drug concentrations (fivefold dilutions) and incubated at 37°C for 1 h. Fifty microliters of a virus suspension normalized for equal numbers of infectious units, as determined by TZM-bl assay (diluted in DMEM containing 10% FBS and 80 μg/ml DEAE-dextran), was then added to the cells. The two RT-inhibiting drugs used in this analysis, 3TC and NVP, were at final concentrations ranging from 0.008 to 5.0 μM. The infected cells were analyzed for luciferase expression after 2 days. Controls included cells exposed to no virus or to virus without drugs. Relative infectivity was calculated by dividing the number of

luciferase units at each drug concentration by values from wells containing virus without drug. Data were fitted with a nonlinear function, and 50% inhibitory concentrations (IC₅₀s) were calculated by least-squares regression analysis. All results were replicated in three separate experiments.

RESULTS

Analysis of *trans*-heterodimeric RT virion packaging and activity. The proviral clone pSG3^{FN} (FN) (Fig. 1A) was used to study the incorporation of heterodimeric *trans*-RT into virions when coexpressed with the bicistronic *vpr-p51/p66* expression plasmid (29). The FN clone was selected for this purpose since it contains a deletion in RT that includes most of the RNase H region and extends 13 amino acids into the carboxyl terminus of the p51 domain (7). This created a defective RT (3, 17), while the *pol* reading frame, including IN, remained open. Rescue of the RT defect by the *trans*-heterodimeric RT requires expression of the two subunits (Vpr-p51 and p66), stable association of the p51 (Vpr-p51) and p66 subunits within the cytosol of the cell, interaction of Vpr with Pr55^{Gag}, incorporation of the Vpr-p51/p66 complex into virions, proteolytic cleavage to liberate Vpr from p51/p66, and proper interaction of RT with the template-primer.

To determine the feasibility of the above strategy, we first tested whether the Vpr-p51 fusion protein could selectively incorporate p66 into virions. For this purpose, 293T cells were cotransfected with *vpr-p51/p66* and FN. Progeny virions were pelleted through a 20% sucrose cushion by ultracentrifugation and analyzed by immunoblot analysis. Using anti-RT MAb, two predominant proteins detected were consistent with the molecular masses of p51 and p66 (Fig. 1B, lane 6) and comigrated with those detected from wild-type SG3 virions (lane 1). Neither protein was detected when RT-negative SG3^{M7} (M7) virions were analyzed (lane 2). Detection of the 51-kDa polypeptide suggested that p51 was liberated from the Vpr-p51 fusion protein by proteolytic processing. However, the molecular mass of unprocessed Vpr-p51 fusion protein (if present) is very similar to that of p66. Therefore, a MAb specific to the RNase H domain of p66 was used as a probe to confirm virion association of the p66 subunit (Fig. 1C, lane 6). To examine whether the presence of p66 in virions was mediated by Vpr-p51, virions were generated by cotransfecting 293T cells with FN and the mutant *vpr-Δp51/p66* expression plasmid, which abrogates expression of the p51 coding region without affecting p66 expression (data not shown). Western blot analysis of virions detected p66, and to a lesser extent p51, indicating non-Vpr-p51 mediated p66 incorporation and subsequent cleavage into the p51 subunit. As additional controls, virions produced by transfecting 293T cells with FN alone and FN in combination with the pLR2P-vprRT (*vpr-p66*) expression plasmid were analyzed. A protein comigrating with p51 that likely represents the truncated RT protein product (p51^{Δ13}) was detected in FN virions. When the *vpr-p66* expression plasmid was cotransfected with FN, p66, p51, and unprocessed Vpr-p66 were detected in virion preparations (lane 4). Immunoblot analysis using a MAb against CA confirmed that approximately the same amount of each virus was analyzed (Fig. 1D).

To examine whether the *trans*-heterodimeric RT could rescue the defect in FN infectivity, the transfection-derived virions were analyzed using the single-cycle TZM-bl reporter as-

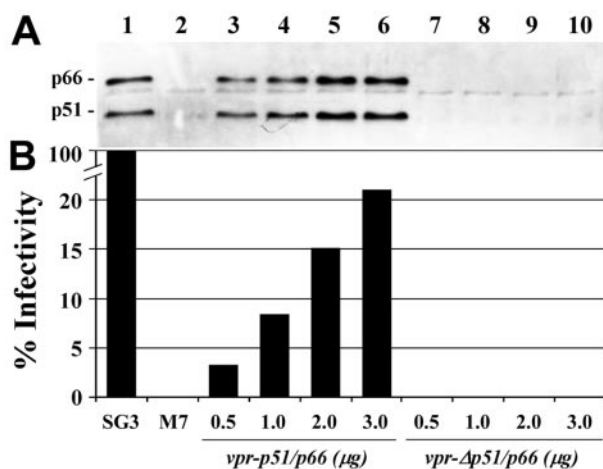


FIG. 2. Analysis of complementation using RT-deficient M7 virus. Increasing DNA concentrations of *vpr-p51/p66* or *vpr-Δp51/p66* (ranging from 0.5 to 3.0 µg) were transfected into 293T cells along with a constant amount of M7 (6 µg) and *vpr-IN* (1 µg). (A) Virion incorporation of *trans*-RT subunits. Transfection-derived virions were concentrated by ultracentrifugation, lysed, and analyzed by immunoblotting using anti-RT MAb (8C4). (B) Analysis of infectivity. Virions were analyzed for infectivity using the TZM-bl reporter cell line. Results are expressed as a percentage of the wild-type SG3 virus.

say. In three independent experiments, cotransfection of the *vpr-p51/p66* expression plasmid rescued FN infectivity to levels of 15 to 20% compared to the wild-type SG3 virus (Fig. 1E, lane 6). Virus derived by cotransfecting 293T cells with FN and *vpr-p66* exhibited a similar level of infectivity (lane 4). The infectivity of FN virion derived by cotransfection with *vpr-Δp51/p66* was approximately 3.5% of wild-type SG3 (lane 5). The RT-defective M7 and FN viruses had no detectable infectivity (lanes 2 and 3, respectively). These results suggested that the heterodimeric *trans*-RT was to catalyze HIV-1 reverse transcription, albeit less efficiently than the endogenous RT.

RT-deleted M7 provirus significantly reduces nonspecific p66 incorporation. Our *trans*-heterodimeric RT strategy for analyzing p51/p66 subunit function necessitated Vpr-p51-mediated incorporation of p66. Nonspecifically packaged p66 likely forms p66/p66 homodimers and through proteolytic processing would generate p51/p66 RT heterodimers, thus confounding subunit-specific analysis. One possible explanation for the nonspecific packaging of p66 observed in Fig. 1 is occasional translational readthrough of the TAA stop codon at the 5' end of p51 in the *vpr-Δp51/p66* expression plasmid. A second possibility is that p66 may incorporate into virions via association with the FN mutant Gag-Pol polyprotein during virion assembly. Therefore, the FN proviral DNA construct was substituted with M7, which has multiple mutations in the RT and IN coding regions and does not encode RT and IN, including that which conceivably could be generated via intermolecular genetic recombination with the *vpr-p51/p66* plasmid. Virions generated by cotransfection of M7 with increasing concentrations (0.5 to 3.0 µg) of the *vpr-p51/p66* expression plasmid were probed with anti-RT MAb. Increased detection in virion-associated p51 and p66 correlated with increased amounts of transfected *vpr-p51/p66* plasmid DNA (Fig. 2A, lanes 3 to 6). In contrast to our findings with FN (Fig. 1),

virions generated by cotransfecting 293T cells with M7 and the mutant *vpr-Δp51/p66* expression plasmid did not contain detectable p66 (lanes 7 to 10). Wild-type SG3 virions showed normal RT (p51 and p66), while M7 lacked RT (lanes 1 and 2, respectively). Probing a replica blot with MAb against CA confirmed that approximately the same amount of each virus was analyzed (data not shown). These results demonstrated that the p66 RT subunit is specifically incorporated into M7 virions by the Vpr-p51 fusion protein.

To determine if the heterodimeric *trans*-RT was functional, M7 was cotransfected into 293T cells along with *vpr-IN* and increasing concentrations of *vpr-p51/p66*. The *vpr-IN* expression plasmid was included since the M7 clone does not express the IN protein. IN is required for integration of viral cDNA, which allows analysis using the TZM-bl reporter cell line and for efficient initiation of reverse transcription (30, 50). Relative virus infectivity was found to correlate with increased packaging of the *trans*-RT (Fig. 2B, lanes 3 to 6). Infectivity at concentrations greater than 3.0 µg of *vpr-p51/p66* was slightly reduced (data not shown), probably because both the total DNA concentrations used for transfection and the amount of protein that can be packaged into virions can be a limiting factor. Virions generated by cotransfection of M7, *vpr-IN*, and *vpr-Δp51/p66* (0.5 to 3.0 µg) were 0.05% or less infectious compared to SG3. The wild-type SG3 infectivity was normalized to 100% (lane 1). These results demonstrate that the heterodimeric *trans*-RT is functional, and by using the M7 proviral clone, negligible complementation of virus infectivity is detected as a result of non-Vpr-p51-mediated packaging of p66.

Effect of expressing p66 and IN in cis. Our results indicate the rescue of M7 infectivity to a maximal level of approximately 15% compared to wild-type SG3. This may be explained, at least in part, by reports showing defects in virions lacking RT-IN expression and packaging as a contiguous protein, including aberrant morphology and RNA dimer conformation (35, 45, 51). In an attempt to enhance the complementation efficiency of our assay, a *vpr-p51/p66-IN* expression plasmid was constructed and cotransfected into 293T cells with M7. Progeny virions exhibited decreased infectivity (Fig. 3A, lane 4) compared to virions complemented with *vpr-p51/p66* (lane 3). Virions concentrated by ultracentrifugation were analyzed by immunoblotting using the p66 (RNase H)-specific MAb. The *vpr-p51/p66*-complemented virions (Fig. 3B, lane 3) incorporated p66 at levels comparable to wild-type SG3 (lane 1), while the RT-negative M7 virions showed no p66 (lane 2). Virions generated by cotransfection of M7 and *vpr-p51/p66-IN* (lane 4) had reduced p66 compared to *vpr-p51/p66*-derived virions (lane 3) and also showed unprocessed p66-IN (RT-IN). Probing a replica blot with MAb to CA confirmed that approximately the same amount of each virus was analyzed (Fig. 3C).

Effect of expressing p66 and Vpr-p51 from separate plasmids. We examined the efficiency of complementation when the Vpr-p51 and p66 subunits were expressed from separate mRNAs in the transfected cells. 293T cells were cotransfected with M7, pLR2P-vpr-p51 (*vpr-p51*), pLR2P-p66 (*p66*), and *vpr-IN*, and progeny virions were analyzed. Infectivity was rescued to a level similar to that exhibited previously using *vpr-p51/p66*, about 10 to 13% of wild-type SG3. Complementation analysis using either *vpr-p51* or *p66* only rescued infectivity by 0.1% and

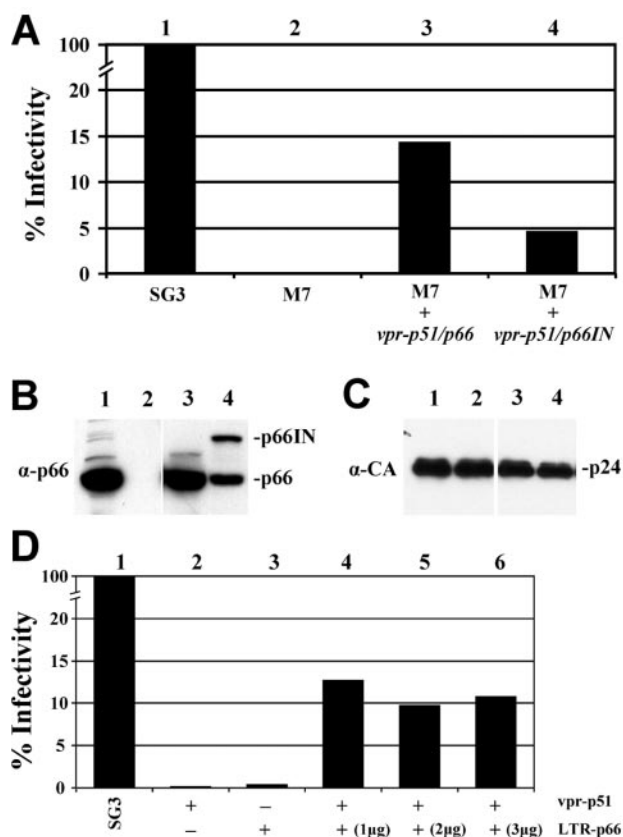


FIG. 3. Alternative approaches for *trans*-heterodimeric RT complementation. (A) M7 virions derived by cotransfection with *vpr-p51/p66* and *vpr-IN* or *vpr-p51/p66-IN* were analyzed for infectivity by the TZM-bl assay. Results are expressed as a percentage compared to the wild-type SG3 virus. (B and C) Immunoblot analysis. The virions were examined for (B) p66 and (C) CA using a MAb specific for either the RNase H subdomain (7E5) or CA, respectively. (D) Infectivity analysis of virions generated by expressing two monocistronic RT constructs, *vpr-p51* and *LTR-p66*. The plus and minus signs indicate the presence and absence of the plasmid included in the cotransfection, respectively. The amount of *vpr-p51* used was kept constant (3 μg), while *LTR-p66* was transfected in increasing amounts (1, 2, and 3 μg), as indicated in parenthesis. M7 and *vpr-IN* were also included in the transfection. The transfection-derived virions were analyzed for infectivity using TZM-bl cells.

0.2% of wild-type SG3, respectively (Fig. 3D, lanes 2 and 3). These results indicate rescue of M7 virion infectivity when p66 and Vpr-p51 are coexpressed from separate mRNAs. Expression of Vpr-p51 and p66 from separate genetic elements will facilitate the manipulation of this approach for analyzing RT function, since this allows the ratios of the two plasmids to be varied.

Distinction between Vpr-p51 and p66 on immunoblots. The *vpr-p51/p66* expression cassette places *vpr* and RT in frame and preserves the N-terminal PR cleavage site of RT by including 11 amino acids of PR (11Pro) between Vpr and RT (29). Thus, the molecular mass of the unprocessed Vpr-p51 fusion protein is indistinguishable from that of p66 when analyzed by Western blotting. This has necessitated the use of a MAb specific to the RNase H domain for specific detection of p66 in virions. To distinguish between these two proteins (Vpr-p51 and p66) by

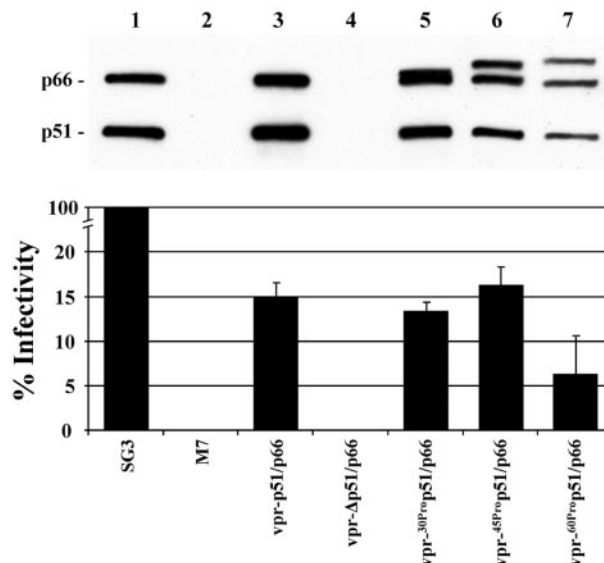


FIG. 4. Distinction between Vpr-p51 and p66 by molecular mass. (A) Immunoblot analysis of virions derived by transfecting 293T cells with M7 and *vpr-p51/p66* expression plasmids containing the different-sized Pro coding sequences. The 8C4 MAb was used as a probe to detect both the p51 and p66 subunits. (B) The transfection-derived virions were examined for viral infectivity using TZM-bl cells. Results are expressed as a percentage of the wild-type SG3 virus.

molecular mass, PR sequence encoding 30, 45, or 60 amino acids was introduced between the Vpr and p51 coding regions in *vpr-p51* (*vpr-30Pro-p51/p66*, *vpr-45Pro-p51/p66*, and *vpr-60Pro-p51/p66*, respectively). Immunoblot analysis of virions derived by cotransfection of *vpr-30Pro-p51/p66* along with M7 into 293T cells showed that adding 30 amino acids of PR was not sufficient to clearly differentiate Vpr-p51 and p66 (Fig. 4, lane 5). However, the addition of 45 or 60 PR residues allowed a clear distinction between Vpr-p51 and p66 (lanes 6 and 7). Analysis of infectivity for the 30Pro- and 45Pro-derived *trans*-RT-containing virions indicated that they rescued M7 infectivity at levels comparable to the original (11Pro-containing) *vpr-p51/p66* construct. In contrast, the 60Pro-containing construct rescued infectivity less efficiently (lane 7). Taken together, these results indicate that *vpr-45Pro-p51/p66* is a viable alternative to the original *vpr-p51/p66* construct for analyzing *trans*-RT heterodimer structure/function. Specific detection of Vpr-p51 and p66 based on molecular mass will facilitate quantitative analyses of the heterodimer.

Chemotherapeutic inhibition of the *trans*-RT heterodimer.

The *trans*-heterodimeric RT assay may be of clinical relevance for analyzing HIV-1 RT inhibitors, drug resistance, and the effects of drug resistance mutations on viral fitness. To examine the response of the *trans*-heterodimeric RT to anti-RT drugs, transfection-derived SG3 virions and *vpr-p51/p66*-complemented virions were used to infect TZM-bl indicator cells in the absence or presence of either 3TC or NVP (0.008 to 5.0 μM). Both drugs exerted a potent, dosage-dependent antiviral effect, as evidenced by an inhibition of infectivity. The IC₅₀s for SG3 and *vpr-p51/p66* complemented virions treated with 3TC were 0.716 and 1.207 μM, respectively. The IC₅₀s for these virions treated with NVP were 0.187 and 0.046 μM, respec-

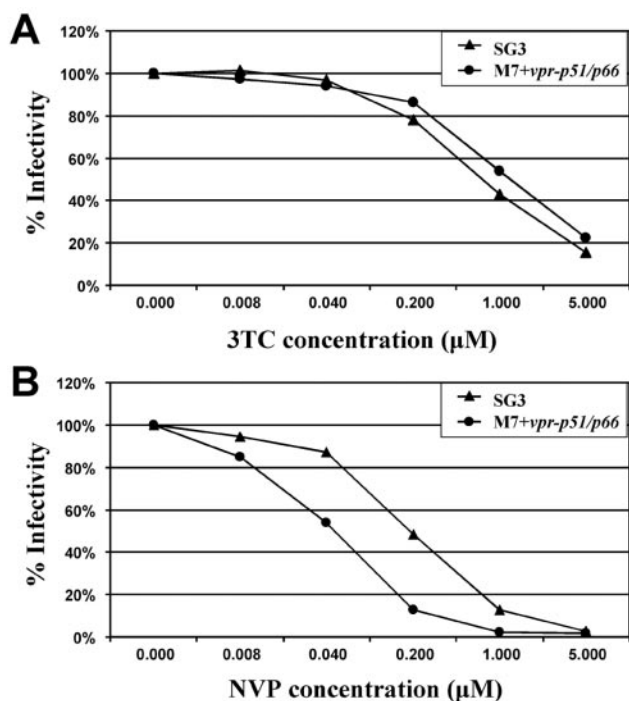


FIG. 5. Inhibition of *trans*-RT using NRTI and NNRTI. Virions derived by transfection with proviral SG3 or cotransfection with M7, *vpr-p51/p66*, and *vpr-IN* were used to infect the TZM-bl reporter cell line in the presence of 0.008 to 5.0 μ M (A) 3TC or (B) NVP, and inhibition of infectivity was determined as described in Materials and Methods. The results from one representative experiment are shown. Reproducibility was confirmed by repeating the assay in duplicate in three independent experiments. The infectivity results are expressed as a percentage of the non-drug-treated virus. Data were fitted with a nonlinear function, and IC_{50} s were calculated by least-squares regression analysis.

tively. These results indicate that the effect of NRTIs on the *trans*-heterodimeric RT is similar to that observed when RT is expressed in its native context (Fig. 5). Interestingly, the IC_{50} s of NVP were approximately fivefold greater for SG3 provirus compared to *trans*-RT-complemented virions. This observation is consistent with other NNRTIs analyzed (data not shown). A possible explanation may be the effects of NNRTIs on Pol maturation/processing (48).

DISCUSSION

The *trans*-heterodimeric RT assay described in this report will help to generate new, physiologically relevant insights furthering our understanding of RT. Extensive analyses of RT function have been carried out using biochemical and other nonphysiological methods. The difficulties in studying RT function using infectious virus have been twofold. First, mutations in HIV-1 polypeptides (Gag and Gag-Pol) have pleiotropic effects on the virus life cycle, including impairment of assembly, maturation, etc. (31, 35, 52). Second, since both RT subunits are derived from the same coding region, the relative arrangements of their subdomains differ markedly (41), and thus, a mutation in one subunit is structurally and functionally nonequivalent to the same mutation in the other subunit. Both

of these characteristics have hindered detailed molecular analysis of the individual subunits in the context of infectious virions and human target cells. Our studies with the FN provirus indicate that the p66 subunit expressed by *vpr-p51/p66* was, at least in part, incorporated into progeny virions via a non-Vpr-p51-mediated (nonspecific) mechanism. To test if the truncated RT or IN protein expressed in *cis* as part of the FN Gag-Pol polypeptide was responsible for nonspecific incorporation, we used the M7 clone that does not express RT and IN. *trans*-complementation analysis with M7 suggested that blocking RT-IN expression from provirus significantly reduced nonspecific p66 incorporation. Our ability to package the Vpr-p51/p66 complex into M7-derived virions in a Vpr-p51-dependent manner, with minimal non-Vpr-p51-mediated packaging of p66, subsequent proteolytic processing to remove Vpr, and generation of a functional RT heterodimer will allow new possibilities for analyzing p51 and p66 in a context that is more physiologically relevant to virion assembly and host cell infection compared to recombinant proteins expressed in *Escherichia coli*. This approach may also provide insights into other viral and host cell factors that affect reverse transcription, either as an enzymatic process or in the context of the reverse transcription complex.

In normally assembled HIV-1 particles, the amount of RT packaged into virions is a function of Gag-Pol expression. It is quite clear that the amount of RT packaged via fusion to Vpr is greater than the amount naturally packaged. It seems notable that the infectivity of RT-negative virions complemented with the *trans* heterodimer (p51/p66) is only rescued to approximately 15 to 20% compared to the wild-type virus. This is consistent with previous studies that reported that the absence of a contiguous RT-IN protein causes multiple defects, including decreased infectivity and atypical virion morphology, RNA conformation, and core morphogenesis (35, 45, 51). Moreover, complementation analysis of a complete provirus (encoding full-length Gag-Pol) containing a catalytically inactive RT (D185N) with *vpr-p51/p66* only restores infectivity to about 15 to 20%, suggesting that the presence of a catalytically active RT as a part of Gag-Pol (RT-IN) may be important for efficient rescue (unpublished observations). This suggests that providing RT in the correct context (as RT-IN) generates more infectious viral particles and that the quantitative amount of RT packaged, within certain limits, is less relevant for determining the efficiency of infection.

The *vpr-p51/p66-IN* expression plasmid was constructed in an attempt to improve complementation efficiency by the *trans*-RT heterodimer. This strategy provided full-length RT-IN (p66-IN) containing both catalytically active RT and IN. However, our analysis showed that the p66-IN fusion protein did not increase complementation efficiency. On the contrary, a decrease in infectivity was detected. Multiple factors could explain this observation. First, Vpr-p51/p66-IN dimerization may impair processing at the RT-IN cleavage site. This could imply that the RT-IN cleavage site is only recognized by the viral PR when RT exists in a homodimeric form, either as a precursor or as a mature protein (p66). Second, It is also possible that Vpr-p51-associated p66-IN (Vpr-p51/p66-IN) is not properly folded. Third, the Vpr-p51/p66-IN and/or p66-IN fusion proteins may exert a dominant-negative effect that affects infectivity. Despite the factors that limit the efficiency of

complementation with *trans*-heterodimeric RT (Vpr-p51/p66), an efficiency of rescue of approximately 15%, along with a background under 0.05% compared to the wild-type virus, allows sufficient sensitivity for subunit-specific structure/function studies using this approach.

Although p51 has been suggested to primarily function as a scaffold to maintain the active structure of p66 (8, 16), other functions have been suggested, including involvement in binding the tRNA^{Lys3} primer (3, 17), loading of p66 onto the template-primer (12), initiation of reverse transcription (3), and enhancement of strand displacement (1, 14). Since HIV-1 RT is functional only as a heterodimeric enzyme, interfering with the heterodimerization process has been proposed as a possible target for therapeutic intervention (28, 38). Mutations in p51 have been implicated in resistance to NNRTIs and inhibitors of RNase H activity. The E138K mutation, which confers resistance to TSAO [2',5'-bis-*O*-(*tert*-butyldimethylsilyl)-3'-spiro-5''-(4''-amino-1'',2''-oxathiole-2'',2''-dioxide)] has been mapped to the p51 subunit (4). The C280S mutation in RT causes resistance to the RNase H inhibitor *N*-ethylmaleimide (24). Similar studies have also identified subunit-specific properties related to the p66 subunit. Some of these include the polymerase and RNase H enzymatic active sites (13, 23, 26), deoxynucleoside triphosphate binding (5, 42), polymerase and RNase H primer grips (9, 33, 36), strand displacement, and processivity (20, 34). Several studies have demonstrated that mutation of amino acids involved in p51-p66 interactions affect RT dimerization and function (9, 30, 47). However, the roles of specific residues and motifs in the individual RT subunits remain largely undetermined. The virus-cell-based strategy described in this report should provide important insights into HIV-1 RT subunit-specific structure/function.

More recently, we have reported on our ability to analyze subunit-specific RT function using infectious virions in human T cells (30), which should augment efforts to understand HIV-1 pathogenesis, drug resistance, and viral fitness mechanisms. Several mutations that confer resistance to currently available NRTI and NNRTI drug therapies have been reported (18). In addition, increased resistance in the presence of specific mutations has also been reported for several anti-RT drugs that are under development (2, 4). Many residues in the proximity of those known to influence RT heterodimer stability have been found to mutate in the presence of both NRTIs and NNRTIs. Residues M230 and P236, which confer NNRTI resistance (18), are within the proximity of L234, a residue known to significantly affect RT dimer stability (9). Similarly, Q151, a residue that mutates under NRTI treatment, is believed to confer resistance by its presence in the p66 subunit (44). However, the same residue in p51 has been reported to interact with other residues that affect p51-p66 interactions (29). Another residue, P294, has been reported to mutate along with the NRTI resistance-conferring mutations at M184 (11). Although the mutation of P294 was not directly related to resistance (11), it is interesting that P294 has also been reported to influence subunit interactions and RNase H activity (43). Using subunit-specific mutagenesis, it should be possible to dissect the roles of these mutations in conferring resistance, altering viral fitness, and influencing other viral processes. We anticipate that this novel RT assay has utility for analyzing RT from both a molecular and a clinical perspective.

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